Ribonucleotide Reductase Regulation in Response to Genotoxic Stress in Arabidopsis

Hélène Roa, Julien Lang, Kevin M. Culligan, Murielle Keller, Sarah Holec, Valérie Cognat, Marie-Hélène Montané, Guy Houlné, and Marie-Edith Chaboute

Institut de Biologie Moléculaire des Plantes du Centre National de la Recherche Scientifique, Université de Strasbourg, 67084 Strasbourg cedex, France (H.R., J.L., M.K., S.H., V.C., G.H., M.-E.C.); Department of Molecular, Cellular, and Biomedical Sciences, University of New Hampshire, Durham, New Hampshire 03824 (K.M.C.); and CEA, IEBE, SBVME, Centre National de la Recherche Scientifique-UMR6191, Université de La Méditerranée, Laboratoire de Génétique et Biophysique des plantes, 13288 Marseille cedex 9, France (M.-H.M.)

Ribonucleotide reductase (RNR) is an essential enzyme that provides dNTPs for DNA replication and repair. Arabidopsis (Arabidopsis thaliana) encodes three AtRNR2-like catalytic subunit genes (AtTSO2, AtRNR2A, and AtRNR2B). However, it is currently unclear what role, if any, each gene contributes to the DNA damage response, and in particular how each gene is transcriptionally regulated in response to replication blocks and DNA damage. To address this, we investigated transcriptional changes of 17-d-old Arabidopsis plants (which are enriched in S-phase cells over younger seedlings) in response to the replication-blocking agent hydroxyurea (HU) and to the DNA double-strand break inducer bleomycin (BLM). Here we show that AtRNR2A and AtRNR2B are specifically induced by HU but not by BLM. Early AtRNR2A induction is decreased in an atr mutant, and this induction is likely required for the replicative stress checkpoint since rnr2a mutants are hypersensitive to HU, whereas AtRNR2B induction is abolished in the rad9-rad17 double mutant. In contrast, AtTSO2 transcription is only activated in response to double-strand breaks (BLM), and this activation is dependent upon AtE2Fa. Both TSO2 and E2Fa are likely induced by single-stranded DNA, present at stalled replication forks or persisting repair intermediates. In contrast, ATM plays a major role in response to DNA double-strand breaks (DSBs), as ATM is directly activated by protein bindings to broken DNA ends. Though DNA damage pathways are conserved among eukaryotes, the transcriptional response induced by genotoxins is primarily regulated in yeast (Saccharomyces cerevisiae) by the ATR ortholog MEC1, whereas this response is primarily ATM dependent in mammals (Elkon et al., 2005). Similarly, the DSB transcriptional response is regulated by ATM in Arabidopsis (Arabidopsis thaliana), as determined by complete transcriptome analyses (Culligan et al., 2006; Ricaud et al., 2007), while the ATR-mediated response to replicative stress was only partially characterized (Culligan et al., 2004). However, these experiments employed very young Arabidopsis plantlets ranging from 5 to 7 d postgermination (Culligan et al., 2004, 2006; Ricaud et al., 2007). The DNA damage response is also controlled by checkpoint proteins that lead to specific cell-cycle arrests as well as changes in the chromatin structure at the site of DNA damage. For instance, Arabidopsis ATR regulates a G2-phase cell-cycle checkpoint, in response to DNA damage and replication inhibitors (Culligan et al., 2004). In addition, the
replication inhibitor hydroxyurea (HU), which inhibits ribonucleotide reductase (RNR)-dependent production of dNTPs for DNA synthesis, appears to induce a novel G1 checkpoint in 5-d-old plantlets (Culligan et al., 2004). Other checkpoint proteins were also identified in Arabidopsis, such as AtRAD17 and AtRAD9 (Heitzeberg et al., 2004) that are epistatic in the DSB response. An ATM-dependent transcriptional regulation of AtRAD17 was also shown in response to γ-irradiation (Culligan et al., 2006; Ricaud et al., 2007).

RNR regulation is of particular interest since it provides the dNTP pool needed for DNA replication and DNA repair. RNR is a heterodimeric enzyme composed of two R1 regulatory and two R2 catalytic subunits. Eukaryotic cells have developed several surveillance mechanisms to regulate RNR activity in response to genotoxic stress to ensure balanced dNTP pools for high-fidelity DNA repair. In yeast, the two genes encoding the catalytic subunits (RNR2 and RNR4) as well the gene encoding the regulatory subunit (RNR3) are induced through Mec1-dependent Rad53 signaling in response to DNA damage (Elledge et al., 1993; Huang and Elledge, 1997; Mulder et al., 2005; Fu and Xiao, 2006). Mammals also express R2 and an alternative R2 termed p53R2. While the former paralog is not induced by DNA damage, p53R2 is activated by p53 in an ATM/CHK2-dependent manner (Tanaka et al., 2000).

In contrast with yeast and mammals, regulation of the small RNR multigene family in response to geno-

Figure 1. Characterization of the AtR2 proteins in Arabidopsis. A, Alignment of the Arabidopsis R2 proteins was performed as described in Supplemental Materials and Methods S1. Numbering of amino acids starts with the first Met of the R2B sequence. Identical amino acids are boxed in black and amino acids with similar physical properties are boxed in white. Functional sites were reported for the sequences as follows: triangles for residues involved in the interaction of R2 with R1, stars for residues required for iron binding and the subsequent generation of the (Fe)2-Y cofactor required for catalysis, and dots for the residues needed for tyrosyl radical. In R2B, R2 residues changed or lost are indicated by white symbols and a putative mitochondrial signal sequence is underlined. B, Phylogenetic tree of R2 proteins. The tree was constructed as described in Supplemental Materials and Methods S1. The scale indicates the evolutionary distance (number of substitution per site). The relevant protein sequences were downloaded from The Arabidopsis Information Resource database for Arabidopsis sequences (TSO2, AT3G27060; AtR2A, AT3G23580; AtR2B, AT5G40942), from Genpept for yeast (ScR2, AAA34988; ScR4, AAB72236), tobacco (NrR2, CA63194), mouse (MmR2, NP_033130; MmR2-P53, NP_955770), B. taurus (BtR2, XP_584910; BtR2-P53, XP_607398), Gallus gallus (GgR2-1, ENSGALP00000030966, GgR2-2, ENSGALP00000026474, GgR2-3, ENSGALP000000258083), rice (OsR2, NP_001055522; OsR2-2, NP_001055568), and Glycine max (GmR2, AAD33202).

www.plantphysiol.org on August 15, 2017 - Published by Downloaded from Copyright © 2009 American Society of Plant Biologists. All rights reserved.
toxic stress in plants is not yet fully understood. For example, among the three Arabidopsis genes encoding the small subunit (AtTSO2, AtRNR2A, and AtRNR2B; Wang and Liu, 2006), AtTSO2 was shown to be strongly induced by ionizing radiation (IR; Culligan et al., 2006; Ricaud et al., 2007) or bleomycin (BLM) plus mitomycin C (MMC; Chen et al., 2003). By contrast, AtRNR1 encoding the large subunit is up-regulated in the DSB response and upon UV-B irradiation (Culligan et al., 2004, 2006; Ricaud et al., 2007). NtRNR1a and NtRNR1b genes are induced by HU in proliferating tobacco (Nicotiana tabacum) cells but are differentially expressed with a high and low induction of NtRNR1a and NtRNR1b, respectively (Chaboute et al., 2002; Lincker et al., 2004). In addition, NiRNR1a is also up-regulated by UV-C, and E2F cis-elements present on its promoter are important to drive its specific induction (Lincker et al., 2004). However, functional studies showing the direct implication of E2F in the RNR DNA damage response have not yet been demonstrated in Arabidopsis. In addition to the partial characterization of AtRNR gene response to DNA damage, recent data showed that the tso2-rrn2a double mutant displays genomic instability with selective induction of DNA repair genes, and is hypersensitive to UV-C (Wang and Liu, 2006). However no clear link was established between RNR induction and DNA damage signaling.

The aim of this article is to characterize the RNR gene response to the replication-blocking agent HU and the DSB inducer BLM in plants at 17 d postgermination, expressing high levels of the S-phase H4 marker gene. Based on our results, we provide (1) evidence for a specific induction of AtRNR genes with respect to genotoxins, (2) functional analyses of rrr mutants linked to specific sensitivity to genotoxins, and (3) evidence for the AtTSO2 DNA damage response controlled by AtE2Fa. In addition, we highlight a differential AtTSO2 DSB response in the atr mutant, which is dependent upon growth stage and H4 histone gene expression.

RESULTS

The RNR Gene Family in Arabidopsis: RNR2 Gene Diversity Is Conserved through Evolution

The diversity of RNR2 genes in mammals and yeast is linked to specific gene expression in response to genotoxins. To determine the evolutionary link between R2 proteins, we conducted a phylogenetic analysis using Arabidopsis, yeast, and mammal R2 proteins.

Arabidopsis (var. Columbia, ecotype Columbia-0 [Col-0]) genome contains three RNR2 genes, AtTSO2 (At3g27060), AtRNR2A (At3g23580), and AtRNR2B (At5g40942), encoding R2 catalytic subunits (TSO2, R2A, and R2B; Wang and Liu, 2006). However only one RNR1 gene, termed AtRNR1 (At2g21790), encodes the R1 regulatory subunit. Alignment of the R2-encoded proteins revealed that AtR2B is truncated in the N-terminal region and some residues involved in the catalytic function of the enzyme are missing compared to AtR2A or AtTSO2 (Chaboute et al., 2002; Lincker et al., 2004).
1998) highlighted in Figure 1A, five residues are either modified into nonconservative amino acids or absent in the N-terminal half of R2B: one residue (white triangle) involved in the association with R1, three residues (white star) required for iron binding and the subsequent generation of the (Fe)$_2$-Y cofactor required for catalysis, and one residue (white dot) needed for tyrosyl radical. Similarly in yeast R4 (Wang et al., 1997), five functional residues are changed: one residue involved in the interaction with R1, three residues involved in the iron center, and one residue providing the tyrosyl radical (Huang and Elledge, 1997).

Arabidopsis R2 proteins were phylogenetically compared to other known R2 proteins (Fig. 1B). These proteins are divided into two families, one with R2B and TSO2 proteins, and the other with R2A protein. In rice (Oryza sativa), two R2 genes have been identified belonging to the same family as for the two R2 proteins (R2 and R4) in yeast (Saccharomyces cerevisiae [Sc]; Wang et al., 1997). In contrast, in mammals (Bos taurus [Bt], mouse [Mm], and human [Hs]), the two R2 proteins (R2-R2p53) have diverged into two separate families (Tanaka et al., 2000). Even in the same family, significant divergence is apparent between R2 members as for R2B and TSO2 in Arabidopsis or R2 and R4 in yeast.

**Phenotypic Characterization of the Response to Genotoxic Stress**

We previously showed that RNR gene expression is primarily induced during S-phase where DNA replication occurs (Chaboute et al., 2000, 2002). According to this, we investigated whether there are differential AtRNR responses to DNA damage in plantlets enriched in S-phase cells, characterized as having high

![Figure 3. RNR gene response to HU in Arabidopsis plantlets. A, Seventeen-day-old plants treated with HU (1 mM). Gene expression was evaluated in wild type (WT), atm, and atr at different time points during genotoxic treatment. Relative expression of the four AtRNR genes (AtRNR1 [white diamonds], AtTSO2 [black triangles], AtRNR2A [black squares], AtRNR2B [white squares]) was quantified by RT-quantitative PCR (as described in Supplemental Materials and Methods S1) performed on plantlets RNAs. B, HU response of AtRNR2B performed as in A in wild type (white diamonds), rad9 (black squares), rad17 (black triangles), and rad9-17 mutants (black diamonds). sos are indicated. C, Test of hypersensitivity of the mr2a and wild-type plantlets to increasing concentrations of HU (1, 3, and 6 mM). Plants were compared 15 d after germination on HU versus control plants (C).](image-url)
H4 histone gene expression (Reichheld et al., 1995, 1998; Meshi et al., 1998). Studies of the DNA damage response in Arabidopsis plants have typically only included young seedlings (Culligan et al., 2004, 2006; Molinier et al., 2005; Ricaud et al., 2007) in which H4 gene expression was considerably lower than in older plantlets (17-d-old plantlets; Supplemental Fig. S1A). In this plant developmental context, where endoreduplication level was also higher (Supplemental Fig. S1B), we wanted to determine if H4 gene expression is affected by genotoxins. To address this, we analyzed H4 (At5g59970) mRNA levels in 17-d-old plants treated with HU or BLM. HU blocks DNA replication by inhibiting RNR-dependent production of dNTPs required for DNA synthesis, while BLM primarily induces DSBs through generation of oxidative damage. The relative mRNA levels (treated versus untreated plants) were evaluated by semiquantitative PCR, using 18S RNA as a standard. For comparison, we tested if these expression pattern modulations were similar in atm and atr to determine ATM- and ATR-dependent effects on the cell cycle gene.

HU treatment of wild-type plants (Fig. 2A), resulted in a rapid H4 gene induction (0.5 h, 2.3-fold) that was reduced in atr (Fig. 2A), but not in atm where expression increased continuously until 8 h. Interestingly, HU (1 mM) sensitivity tests revealed no difference in the root growth between wild type and atm, but enhanced leaf development was observed in atm (Fig. 2C). This latest phenotype may be accounted for by a stimulation of endoreduplication processes in the leaves of HU-treated atm plants due to the continuous transcriptional activation of H4 that we observed (Fig. 2A). To test this hypothesis, we analyzed endoreduplication level in the leaves of wild-type and atm plants treated with HU or untreated. Indeed, fluorescence-activated cell sorting analyses revealed a higher relative DNA content (treated versus untreated plants) in atm compared to wild-type plants, notably for the 16C DNA content (Fig. 2D). However, this result was never observed in 8-d-old plantlets (data not shown).

Seventeen-day-old wild-type plants treated with BLM (Fig. 2B) showed a strong induction of H4 gene expression (7.5-fold) after 0.5 h. This induction was delayed in atm and considerably reduced in atr. By contrast, we never observed any up-regulation of H4 gene upon HU or BLM treatments in younger plantlets (8-d-old; Fig. 2, A and B, small inset graphs). We suggest that this differential response is linked to plant development, with a lower level of H4 gene expression in 8-d-old plantlets compared to 17-d-old plantlets without genotoxins. Taken together, our results highlight an early transient H4 induction in response to HU and BLM treatments and that this induction is ATR dependent.

RNR Replicative Stress Response

Similar to the regulation of H4, AtrRNR2A, and AtrRNR2B displayed an early induction (0.5 h, approximately 5- to 6-fold) in HU-treated wild-type plants (Fig. 3A). In contrast, no AtTSO2 induction was observed whereas AtRNR1 induction was delayed (>8 h).

In HU-treated atm plants, only the early induction of AtrRNR2B was decreased, showing that functional ATR cannot maintain the maximal induction observed in wild-type plants. However, AtrRNR2A, AtrRNR2B, and AtrRNR1 displayed a late reinduction (6 h) that was lost in atr (Fig. 2A). This late HU-mediated induction may reflect S-phase-specific gene transcription for endoreduplication process controlled by ATR in the absence of genotoxins.
of ATM, as previously suggested for AtH4 gene regulation.

In contrast, the early induction of AtRNR2A was decreased in atr when grown on HU. To understand the physiological relevance of RNR2A induction, we analyzed the hypersensitivity of the mnr2a mutant to HU. The mutant plants grew slower compared to wild type at low HU concentration (1 mM) and died or did not germinate at higher concentrations (3 and 6 mM, respectively; Fig. 3C), demonstrating the importance of R2A in the replicative stress response in Arabidopsis. Since HU-dependent AtRNR2B induction was not affected in atr (Fig. 2A), we investigated the role of the AtRAD9 and AtRAD17 checkpoint proteins, which we showed to be involved in the replicative stress response according to their high sensitivity to HU (Supplemental Fig. S2A). HU AtRNR2B induction was lost in the single rad9 and rad17 mutants as well as in the rad9/rad17 double mutant (Fig. 3B), highlighting the RAD9/RAD17-mediated induction of AtRNR2B in the replicative stress response. In contrast, AtTSO2 was up-regulated in atr, possibly through a transcriptional derepression.

These data suggest that each subunit employs a unique expression pattern in response to the HU-induced S-phase checkpoint, and this expression is sometimes dependent upon functional ATR, ATM, or RAD9/RAD17.

RNR BLM Response

Although neither AtRNR2A nor AtRNR2B were induced in wild-type plants treated with BLM (Fig. 4A), we observed under the same conditions a significant AtTSO2 induction from 1.5 to 8.5 h of treatment. The AtRNR1 gene was also induced at 6 to 8.5 h, but to a lesser degree than AtTSO2, and displayed different kinetics of induction. Although AtTSO2 induction was maximal at 3.5 and 8.5 h of the BLM treatment, this could reflect differences in the time course of DSB induction. To determine this, we employed a neutral comet assay (Fig. 4B). This assay shows that generation of DSBs increase exponentially up to about 3.5 h, and reaches a plateau to approximately 8.5 h.

Similar to γ-irradiated young plantlets (5- to 8-d-old; Culligan et al., 2006; Ricaud et al., 2007), an ATM dependency of AtTSO2 and AtRNR1 gene expression was observed in 17-d-old BLM-treated plants (Sup-
To demonstrate that TSO2 induction is related to its involvement in the DSB response, we analyzed the sensitivity of tso2 mutants to BLM. Their sensitivity was higher compared to wild-type plants as BLM concentration was increasing (Fig. 4C). Therefore the specific TSO2 up-regulation induced by BLM suggests that TSO2 is involved in the response to DSBs.

**Specific BLM-Induced Expression of TSO2 in atr**

In atr plants treated with BLM, TSO2 displays biphasic gene induction both early (0.5 h) and late (6–8.5 h; Fig. 5A). This late up-regulation was significantly (3-fold) higher than in wild type (Fig. 5A), and interestingly was never observed in younger material (5- to 8-d-old) treated with BLM (Fig. 5A, left border) or γ-irradiated (Culligan et al., 2006; Ricaud et al., 2007). Although the atr mutation is in a different wild-type ecotype background (Wassilewskija [Ws]), the maximal AtTSO2 induction upon BLM treatment was similar in wild-type Col-0 and wild-type Ws (Fig. 5B).

To explain the DSB response in atr, we hypothesize that more DSBs may occur in 17-d-old plants compared to younger seedlings. Therefore, we quantified the DSBs in wild-type and atr plantlets from 5 and 17 d postgermination using the neutral comet assay. In the absence of BLM (Fig. 5C), we observed no DSB content difference between wild type and atr in 5-d-old plantlets but a significant DSB increase (at least 1.5 more) was revealed in atr compared to wild type (Col-0 and Ws) in 17-d-old plants. This observation suggests that more genomic instability occurs in atr at this developmental stage without BLM treatment. After a 6-h BLM treatment, no significant difference was observed between wild type and atr either in 5- or 17-d-old plants (Fig. 5C), perhaps due to a saturated response from large amounts of DSBs generated. As AtTSO2 induction is ATM dependent, we checked if this induction in atr may be due to ATM up-regulation. Indeed, a
significant induction of ATM was observed in control (C) or BLM-treated atr plants (T) from 17 d postgermination (Fig. 5D), but not in younger plantlets (data not shown). Taken together these data suggest that genomic instability is increased in atr likely due to an up-regulation of AtATM in 17-d-old plantlets. Compared to atr control plants, the level of ATM mRNA is considerably lower in control wild-type plants but in these plants, a 3-fold induction was observed in response to BLM. This may explain the discrepancy observed in the up-regulated expression of TSO2 between atr and wild type upon BLM treatment.

**AtTSO2 Induction Is Controlled by AtE2Fa in the ATM-Mediated BLM Response**

We determined the transcriptional regulation of AtTSO2 and observed similar increased levels between AtTSO2 promoter activity (2-fold) and AtTSO2 mRNA (Qt = 3, Fig. 4A) after a 1.5-h BLM treatment (Supplemental Fig. S3D), suggesting that AtTSO2 is regulated at the transcriptional level in the DSB response. It has been shown that AtE2Fa was induced after BLM plus MMC treatment (Chen et al., 2003) and therefore this transcription factor may be a good candidate for controlling AtTSO2 gene induction in response to BLM treatment.

To test this, we analyzed a T-DNA insertion line for the gene AtE2Fa. The T-DNA insertion occurs in exon 10 of the gene and the sequence of the left-border flanking sequence tag given by GABI-Kat GenBank was confirmed by sequencing the PCR product (Fig. 6A). Southern analysis showed only one T-DNA insertion that contained a deletion of approximately 700 bp on the right border (data not shown). To confirm that we had null mutant lines, AtE2Fa gene expression was analyzed by reverse transcription (RT)-PCR in a homozygous mutant compared to the wild type in 17-d-old plantlets: No expression was observed in the mutant line (Fig. 6B, Supplemental S3A), whereas AtE2Fa induction increased until 3 h in the wild type. Homozygous e2fa −/− plants presented no obvious growth phenotype, but when treated with BLM (10^-6, 10^-5 M), they proved more sensitive to BLM than the wild type (Fig. 6C). Besides their BLM sensitivity, e2fa mutants failed to show any induction of the AtTSO2 gene upon BLM treatment (Fig. 6E). To determine that the lack of TSO2 induction is due to the E2Fa mutation, we demonstrated the T-DNA insertion line with a TAG (Etag) fusion of AtE2Fa. A protein of the correct expected size was detected in western experiments using an antibody directed against the Etag epitope (Fig. 6D). In the complemented mutant (showing a BLM sensitivity similar to wild type; Supplemental Fig. S3B), AtTSO2 expression was rescued in response to BLM (Fig. 6E). This demonstrates the E2Fa-mediated AtTSO2 induction in response to BLM, probably through binding of the E2Fa transcription factor on its target cis-elements present on the TSO2 promoter (Supplemental Fig. S3C). In addition, AtE2Fa gene induction was lost in atm upon BLM treatment but increased in atr (Fig. 6F). Thus, our data suggest that the ATM-mediated transcriptional activation of AtE2Fa is needed to regulate the cellular response to DSBs.

**DISCUSSION**

**AtRNR2 Genes Are Differentially Expressed in Response to Genotoxins**

We have shown here a differential transcriptional response of the three AtRNR2 genes: AtITSO2 is only induced by DSBs and therefore may constitute a transcriptional marker of the DSB response. However, AtRNR2A and AtRNR2B, which are induced in response to HU but not DSBs, represent transcriptional markers of replicative stress (Fig. 3A). As HU is a direct inhibitor of RNR, it is possible that a simple feedback regulation mechanism, independent of single-stranded DNA induction, may occur. The physiological response of rnr2a to HU but not to BLM (data not shown) suggests activation of replicative stress signaling. In contrast, the hypersensitivity of iso2 to BLM but not to HU (data not shown) indicates activation of DSB signaling. A differential gene expression was also observed for RNR1 genes in response to DNA damage in tobacco. Indeed, among the NtRNR1 small multigenic family, we showed a strong induction of the NtRNR1a gene in response to HU compared to NtRNR1b (Chabouté et al., 2002; Lincker et al., 2004).

Ultimately, it appears that through evolution, RNR genes have evolved to fulfill specific functions, notably in DNA repair. Indeed, p53R2 was shown to be induced by both UV and γ-radiation in humans (Tanaka et al., 2000). Alternatively in yeast, induction of RNR2 and RNR4 genes was observed upon various stresses such as γ-radiation (Gasch et al., 2001), HU, and UV (Aboussekra et al., 1996; Huang and Elledge, 1997).

Our data suggest that the specific induction of AtRNR2 genes in response to genotoxic stress, may suggest that
these genes have unique roles in Arabidopsis DNA repair.

**The Transcription Factor AtE2Fa Is Regulated by ATM and ATR in the DNA Damage Response**

Through evolution, RNR gene expression is tightly controlled in response to DNA damage. In yeast, RNR induction is achieved through derepression of CRT1 and CRT10 under the control of the MEC1/DUN1 pathway in response to IR (Huang et al., 1998; Fu and Xiao, 2006). In mammals, some DNA repair genes are controlled by the p53 transcription factor: through RAD51 repression to regulate homologous recombination (Arias-Lopez et al., 2006) or p53R2 induction to produce dNTPs (Tanaka et al., 2000). More recently, the E2F7 and E2F8 transcriptional repressors were shown to act upstream of E2F1, thereby influencing the capacity of cells to initiate a DNA damage response in mammals (Panagiotis Zalmas et al., 2008). In contrast, the AtE2Fa transcriptional activator regulates the expression of AtTSO2 in the Arabidopsis DSB response (Fig. 6E) as well as that of a subset of DNA repair genes harboring E2F elements in their promoters such as AtRAD51 (Supplemental Fig. S4) and AtBRCA1 (data not shown). These E2F target genes are also coexpressed in the DNA repair network (http://atted.jp) including 16 genes such as AtPARP1, AtPAR-like, AtPOL2a, and AtRAD17, and are also ATM-dependent induced in the DSB response (Culligan et al., 2006; Ricaud et al., 2007). As for AtTSO2, the ATM-mediated induction of AtE2Fa may be required for the specific induction of these genes. In contrast, the lack of AtTSO2 induction in the HU response in wild type might be due to the decreased AtE2Fa expression that we observed (Supplemental Fig. S6A). In addition, as AtTSO2 is up-regulated in atr by HU, we cannot exclude a down-regulation of AtE2Fa mediated by ATR, perhaps leading to no AtTSO2 induction in wild type. Similar results were obtained for the AtFAS1 gene encoding the chromatin remodelling factor with HU treatment, with no induction in wild type but an up-regulation in atr (Supplemental Fig. S6B).

This reveals a diversity of mechanisms controlling RNR gene expression between animals and plants in response to DNA damage. Since AtTSO2 as well as AtRAD51 or AtFAS1 are also cell-cycle regulated and target of AtE2Fa (Doutriaux et al., 1998; Vandepoele et al., 2005; Wang and Liu, 2006; Ramirez-Parra and Gutierrez, 2007), this may involve specific coregulators of AtE2Fa controlled by the ATM and ATR pathways in the DNA damage response.

**Model of AtRNR Regulation Linked to Plant Growth and H4 Gene Expression in Response to Genotoxins**

In the absence of a functional ATR in 17-d-old plantlets, where H4 gene expression is high, AtTSO2 gene induction is considerably higher compared to wild type (Fig. 5A) as well as for AtRAD51 (data not shown). A similar expression pattern was observed with IR in 17-d-old plants (Supplemental Fig. S5) but not in 5-d-old plants (Culligan et al., 2006; Ricaud et al., 2007). Compared to BLM, the IR response was increased and occurred earlier in the kinetics. This difference may be due to the fact that BLM is a chemical that needs to be activated before generating DSBs (Liang et al., 2002). AtRNR1 and AtH4 genes are also developmentally regulated in response to HU: These genes are not induced in very young plantlets (Culligan et al., 2004; Fig. 2A for H4), however are significantly up-regulated in wild-type 17-d-old plantlets (Figs. 2 and 3A), but not in atr. Therefore in older plants enriched in endoreduplicated cells, ATR might be important to control the replicative stress response of AtRNR1 and AtH4 genes.

Taken together, these results highlight a complex regulation of RNR genes in the ATM-ATR DNA damage network. On one hand (Fig. 7A), AtTSO2 expression is controlled by an ATM-mediated induction of AtE2Fa in the DSB response that may be negatively controlled by ATR. Indeed, TSO2 expression appears to be also repressed by ATR in the HU response, probably through the down-regulation of AtE2Fa (Fig. 7B). On the other hand the HU response of AtRNR2B is controlled by RAD9/RAD17 and AtRNR2A partly controlled by ATR (Fig. 7B). However, the specific HU response of AtRNR2B is decreased in atm but lost in the rad9/rad17 double mutant, suggesting that ATM may also interfere in the replicative stress response but probably not in the same pathway as RAD9/RAD17.

Since the rad2a mutant is less sensitive than atr to HU (data not shown), R2A and ATR are probably not acting in the same pathway. Interestingly, we observed that the lack of a functional ATM stimulates a late up-regulation of H4 gene as well that of RNR1, RNR2A, and RNR2B genes in response to HU. This may be connected with a developmentally controlled program leading to enhanced endoreduplication. Such a process may require an ATR-dependent DNA replication checkpoint as recently suggested for the function of MIDGET in the topoisomerase VI complex (Kirkik et al., 2007). In addition, the transient H4 up-regulation by HU or BLM may also correspond to a transient S-phase arrest mediated by ATR and linked to the S-phase checkpoint that was never described in younger plantlets (Fig. 2, A and B). This highlights the plasticity of plants in the control of the cell cycle in response to DNA damage throughout development.

**MATERIALS AND METHODS**

**Arabidopsis Lines and Plant Growth Conditions**

Our experiments were performed in various mutants that were already characterized: the atr-3 / - and atm-2 / - null mutants (Garcia et al., 2003; Culligan et al., 2004), the rad9-1 and rad17-1 mutants (Heitzeberg et al., 2004), as well as the bso2-1 / - and rnr2a-1 / - ethyl methanesulfonate mutants.
Seventeen days postgerminated plantlets were used in our experiments. They were grown on Murashige and Skoog medium during 17 d and transferred to plates without (control plants) or with genotoxins (1 mM HU or 10 μM BLM) for 8.5 h in growth chamber. Plants were harvested after 0.5, 1.5, 3.5, 6, and 8.5 h, then frozen in liquid nitrogen.

RNA Extraction and cDNA Synthesis

Total RNA was extracted from plant seedlings with TRIzol (Invitrogen SARL) according to the manufacturer’s instructions. After treatment by Deoxyribonuclease I (Fermentas, UAB), RNAs were stored at −80°C. One microgram of total RNA was then reverse transcribed with the Invitrogen reverse transcriptase (Promega Corporation) using random hexamers as primers.

Real-Time Semi-quantitative PCR and RT-PCR Assays

Amplification was performed with 1 μL of cDNA in a final volume of 25 μL with the qPCR MasterMix Plus for SYBER Green I with fluorescein (Eurogentec), and gene-specific primers (Supplemental Table S1). As a reference for PCR quantification, the 18S ribosomal RNA gene was amplified with specific primers (Supplemental Table S1), but the cDNA was diluted 20-fold more in the PCR reaction. Three quantifications were performed for each sample as described (Supplemental Materials and Methods S1; RT-quantitative PCR). RT-PCR was monitored in 25-μL reactions using GoTaq Flex DNA polymerase (Promega Corporation), 1 μL of cDNA, and the specific primers (Supplemental Table S1). As a reference for PCR quantification, either actin2 or 18S ribosomal primers were used (Supplemental Table S1). Equal volumes of PCR products were analyzed on agarose gels and visualized by ethidium bromide staining. Band intensity was quantified using the software program Quantity One (Bio-Rad).

Mutant Analysis

A T-DNA insertion line for the gene AtE2Fa was available in GABI-Kat genebank (line 348039, see http://www.gabi-kat.de/db/showseq.php?gene= At2g36010). This line was screened for homozygous plants by segregation on (Supplemental Table S1). As a reference for PCR quantification, either actin2 or 18S ribosomal gene was amplified with specific primers (Supplemental Table S1), but the cDNA was diluted 20-fold more in the PCR reaction. Three quantifications were performed for each sample as described (Supplemental Materials and Methods S1; RT-quantitative PCR). RT-PCR was monitored in 25-μL reactions using GoTaq Flex DNA polymerase (Promega Corporation), 1 μL of cDNA, and the specific primers (Supplemental Table S1). As a reference for PCR quantification, either actin2 or 18S ribosomal primers were used (Supplemental Table S1). Equal volumes of PCR products were analyzed on agarose gels and visualized by ethidium bromide staining. Band intensity was quantified using the software program Quantity One (Bio-Rad).

E2Fa-Etag Constructs

The open reading frame of the AtE2Fa cDNA (At2g36010) sequence was amplified by PCR from total cDNA using the specific primers RW2 and FW2 (Supplemental Table S1) and cloned in TOPO vector. A XmnI-XbaI DNA fragment was cloned in frame with Etag in pNEX-1 vector, under the control of the 35 s promoter (kindly provided by Dr. J.-L. Evrard, IBMP). A second digestion was then performed with EcoRI and HindIII for cloning into pGreen0029 vector. Finally, a GV3101 Agrobacterium strain containing the pSOUP plasmid was transformed with the pGreen0029 vector and used for floral-dipping transformation. AtE2Fa migrates with an apparent molecular mass of 66 kD and the addition of the 16 amino acids of Etag does not modify this migration pattern.

ArTSO2 Promoter GUS Construct and GUS Quantification

A PstI-XhoI DNA fragment extending from 1.078 bp upstream and 21 bp downstream of the ATG from TSO2 genomic sequence was cloned into the PstI-XhoI restriction sites of the binary vector pB101. Plasmid construct was introduced into Agrobacterium tumefaciens strain GV3101 and used to transform Arabidopsis. Ten independent transgenic lines were obtained. Quantification of GUS activity was carried out using the Tropix GUS Light kit (Applied Biosystems) as described (Chaboudt et al., 2002).

Plant Protein Extracts and Western Experiments

Plant protein extracts were performed as described (Lincker et al., 2006) and a monoclonal antibody raised against Etag epitope (GE Healthcare, Europe GmbH) was used in western-blots experiments.

Comet Assays

About 20 17-d-old plantlets were incubated with or without BLM and were frozen in liquid nitrogen and stored at −80°C. DSBs were evaluated using neutral comet assay as described (Menke et al., 2001). Dry agarose gels were stained with 15 μL ethidium bromide (5 μg/mL) and were used for evaluation with a Nikon E800 fluorescence microscope. DNA damage in each comet tail was evaluated as described (Collins, 2004), assigning an arbitrary value (0–4) according to the comet size. In each experiment, the sum of 100 comet scores corresponds to arbitrary DNA damage unit. The mean value of four independent slides was presented.

Flow Cytometry Analyses

Fresh plants were chopped with a sharp razor blade in CysStain-UV-ploidy medium and analyzed as described by the manufacturer, using a Cyflow-R ploidy analyzer (Partec). Five independent experiments were performed.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. A, H4 gene expression; B, DNA content in 8- and 17-d-old plantlets.

Supplemental Figure S2. A, Hypersensitivity of rad mutants to HU; B, ATM-dependent expression in response to BLM.

Supplemental Figure S3. A and B, Characterization of e2fa mutant; C and D, analysis of TSO2 promoter.

Supplemental Figure S4. E2Fa-regulated expression of RAD51 in response to BLM.

Supplemental Figure S5. IR response of TSO2 and RAD51.

Supplemental Figure S6. Gene regulation in HU response.

Supplemental Table S1. List of primers.

Supplemental Materials and Methods S1. RT-quantitative PCR and bioinformatics.

ACKNOWLEDGMENTS

We thank Dr. A. Tissier for providing atm mutant and Pr. H. Puchta for giving us rad9, rad17, and rad9/rad17 mutants, and Dr. Wang for the rnr mutants. We are grateful to Dr. J.L. Evrard for providing Etag vector as well as Dr. K. Angelis for introducing us to the comet assay. We thank also J. Menestier for technical assistance and M. Alioua for help in quantitative PCR analyses. We thank Dr. P. Pfeiffer for the critical reading of the manuscript.

Received April 17, 2009; accepted June 28, 2009; published July 1, 2009.

LITERATURE CITED


Ramirez-Parras E, Gutierrez C (2007) E2F regulates FASCIATA1, a chromatin assembly gene whose loss switches on the endocycle and activates gene expression by changing the epigenetic status. Plant Physiol 144: 105–120